# Phosphatidyl-Tris Rather Than *N*-Acylphosphatidylserine Is Synthesized by *Rhodopseudomonas sphaeroides* Grown in Tris-Containing Media†

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ABSTRACT: We have synthesized 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)serine (*N*-acyl-PS) and 1,2-dioleoyl-*sn*-glycero-3-phospho-Tris (phosphatidyl-Tris) and have characterized both phospholipids by their chemical and chromatographic properties, as well as by their IR, $^{13}C$ NMR, and $^{1}H$ NMR spectra. Comparison of these data with those reported for a phospholipid isolated from *Rhodopseudomonas sphaeroides* grown in Tris-supplemented media [Donohue et al. (1982) *Biochemistry 21*, 2765–2773] indicates that *R. sphaeroides* synthesizes phosphatidyl-Tris rather than *N*-acyl-PS.

The facultative photoheterotrophic bacterium *Rhodopseudomonas sphaeroides*, later renamed *Rhodobacter sphaeroides* (Imhoff et al., 1984), has been used extensively for studies on membrane biogenesis and differentiation (Kaplan, 1978). Incorporation of phospholipids into the intracytoplasmic membrane system of a synchronously dividing population of *R. sphaeroides* occurs discontinuously with respect to the cell cycle (Fraley et al., 1979a,b; Lueking et al., 1978), and this discontinuity appears to reflect the bulk transfer of phospholipids to the intracytoplasmic membrane concurrent with cell division (Cain et al., 1981). In attempts to define the kinetic parameters of this phospholipid transfer and incorporation, labeling with [$^{32}P$]orthophosphoric acid was used and a low-phosphate medium was employed in which the phosphate concentration was reduced from 20 to 2 mM and 18 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)[1] was included. No alteration in growth rate was apparent, but the presence of a new phospholipid was observed when *R. sphaeroides* was grown in this low-phosphate medium (Cain et al., 1981).

The phospholipid, which was found to account for up to 40% of the total cellular phospholipids of *R. sphaeroides*, was isolated and was identified as *N*-acylphosphatidylserine (*N*-acyl-PS) by chemical degradation as well as by its IR and $^{1}H$ NMR spectrum (Donohue et al., 1982a). The same phospholipid also accumulated to 28% and 33% of total phospholipid when *Rhodopseudomonas capsulata* and *Paracoccus denitrificans* were grown in media containing 20 mM Tris, whereas wild-type strains *R. sphaeroides* 2.4.1 and RS2 showed no alteration in their phospholipid composition when grown in Tris-supplemented media (Donohue et al., 1982b). Kinetic studies on the accumulation of the new lipid (Cain et al., 1982) and pulse–chase labeling experiments led the authors to propose that *N*-acyl-PS biosynthesis represents a previously undescribed branch of the phospholipid biosynthetic sequence (Cain et al., 1983).

On the basis of the reported chemical and chromatographic properties, we have questioned the identification of the new phospholipid as *N*-acyl-PS and have suggested that Tris itself may participate in phospholipid biosynthesis of *R. sphaeroides* (Schmid et al., 1986). In the present paper, we report data that identify the lipid isolated by Donohue et al. (1982a,b) as phosphatidyl-Tris. This indicates that exogenous Tris can be taken up into this microorganism and used as a substrate in phospholipid synthesis.

## Materials and Methods

*Synthesis of N-Acyl-PS.* PS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine disodium salt) was from Avanti Polar Lipids, Inc. (Birmingham, AL). Fifty milligrams of PS (62 μmol) was dissolved in 8 mL of chloroform/methanol (2:1) and treated with 2 mL of 30 mM HCl to convert it to the free acid. The chloroform phase was blown to dryness, and the PS was redissolved in 3 mL of dry benzene. Oleic acid (NuChek Prep, Elysian, MN) and 1,1′-carbonyldiimidazole (Sigma Chemical Co., St. Louis, MO), 200 μmol each, were reacted in 5 mL of dry benzene for 4 h at room temperature; then this solution was added to the solution of PS, along with 360 μmol of (dimethylamino)pyridine. After maintaining the solution at 40 °C overnight, the volume was reduced to about 2 mL and the reaction mixture was extracted with 30 mL of chloroform/methanol (2:1 v/v) and washed with water containing enough HCl to bring the final pH to about 7. It was found to be important not to have an excess of carbonyldiimidazole, which led to side products probably through activation of the carboxylic acid of the serine moiety. Attempted synthesis with oleoyl chloride produced many side products and virtually no *N*-acyl-PS.

The product was purified by thin-layer chromatography on silica gel H, developed in a solvent of chloroform/methanol/concentrated ammonium hydroxide/water (65:35:5:1 v/v), and eluted from the silica gel with chloroform/methanol/water (30:50:20 v/v). After addition of chloroform and water to effect phase separation, the eluate was neutralized with HCl to convert ammonium salts of the lipid to the free acid. *N*-Acyl-PS was recovered with a final yield of 56%.

*Synthesis of Phosphatidyl-Tris.* Phosphatidyl-Tris was synthesized by transphosphatidylation from phosphatidylcholine with cabbage phospholipase D (Boehringer Mannheim Biochemicals, Indianapolis, IN). The enzyme reaction was carried out in Mallinckrodt anhydrous diethyl ether which had been treated with sodium metal; because the ether did not contain ethanol as a stabilizer, the formation of phosphati-

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[1] Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PS, phosphatidylserine; *N*-acyl-PS, *N*-acylphosphatidylserine; PC, phosphatidylcholine.

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dylethanol was avoided and the yield of phosphatidyl-Tris was thereby increased. Dioleoylphosphatidylcholine (170 μmol) was dissolved in 5 mL of ether, 0.5 mL of 100 mM $CaCl_2$, 5 mL of 1 M Tris, pH 5.6, and 20 mg of cabbage phospholipase D were added, and the mixture was stirred vigorously at 30 °C for 2 h.

The reaction was stopped by the addition of 2 mL of 1.5% EDTA, and the products were extracted with chloroform/methanol (2:1 v/v). Thin-layer chromatography on silica gel H in chloroform/methanol/concentrated ammonium hydroxide/water (65:35:5:1 v/v) showed that phosphatidyl-Tris ($R_f$ 0.4), phosphatidylcholine ($R_f$ 0.3), and phosphatic acid ($R_f$ 0.07) were present in a ratio of 4:1:5. Phosphatidyl-Tris was purified by preparative thin-layer chromatography with the same solvent system and was repurified by using chloroform/methanol/acetic acid/water (65:35:2:2 v/v) to remove a small amount of unreacted phosphatidylcholine.

*Chemical Degradation of N-Acyl-PS and Phosphatidyl-Tris.* 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)-serine (*N*-acyl-PS) and 1,2-dioleoyl-*sn*-glycero-3-phospho-Tris (phosphatidyl-Tris) were diluted to about 10 μmol/mL. Phosphorus was measured on duplicate 10-μL aliquots (Bartlett, 1959). Duplicate 10- and 20-μL aliquots from each sample were mixed with 22.2 μg of methyl heptadecanoate as internal standard and reacted with either 0.2 N NaOH in methanol (45 min at 45 °C) or 5% w/v concentrated HCl in methanol (180 min at 80 °C). The methyl esters were extracted into hexane, washed with water, and then analyzed by gas chromatography in a Packard 428 gas chromatograph equipped with two 12 ft × $^1/_8$ in. i.d. aluminum columns packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, PA). The areas of the sample peaks were compared to the area of the internal standard peak by using a Spectra Physics 4270 integrator.

*Thin-Layer Chromatography.* Glass plates (20 × 20 cm) were spread with silica gel H (Merck) slurried in water or with silica gel G (Merck) in 0.4 M boric acid. Silica gel H plates were used for the purification of lipids and for determination of $R_f$ values of lipids and their degradation products. The boric acid/silica gel G plates were used for two-dimensional chromatography in the solvent systems used by Cain et al. (1983) and Donohue et al. (1982a), i.e., chloroform/methanol/water/ammonium hydroxide (70:30:3:2 v/v) in the first direction and chloroform/methanol/water (65:35:5 v/v) in the second direction. Phospholipids were detected by spraying with molybdic acid in $H_2SO_4$ (Dittmer et al., 1964); the plates were then sprayed with 50% $H_2SO_4$ and charred.

*Infrared Spectroscopy.* Infrared spectra were taken as liquid film on KCl crystals with a Beckman IR 4240 instrument.

*NMR Spectroscopy.* NMR spectra were recorded on a Varian Unity 300 pulse Fourier transform instrument. Spectra were measured with 20–30 mg/mL samples in 5 mm o.d. NMR sample tubes at the ambient probe temperature of 25 ± 1 °C under identical instrument settings. $CDCl_3$ served as solvent and for field frequency locking purposes in the case of $^1H$ NMR (300-MHz) spectra, while $CDCl_3$–$CD_3OD$–$D_2O$ (50:50:15 v/v) served as solvent for $^{13}C$ NMR spectra (75.429 MHz) obtained under broad-band proton decoupling. Chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane, which served as the internal standard in the $^1H$ NMR spectra. In the $^{13}C$ NMR spectra, peaks were referenced with respect to the $CDCl_3$ signal appearing at 77 ppm.

## Results

*Characterization of Synthetic N-Acyl-PS and Phosphatidyl-Tris.* 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)serine and 1,2-dioleoyl-*sn*-glycero-3-phospho-Tris (Chart I) were synthesized, and their structures were confirmed by $^{13}C$ NMR as illustrated in Figures 1 and 2 and summarized in Table I. Interpretation of $^{13}C$ NMR spectra in $CDCl_3$–$CD_3OD$–$D_2O$ (50:50:15 v/v) is simple and straightforward. In this solvent system, a number of synthetic and naturally occurring phospholipids were shown to exist in the nonaggregated state, giving rise to well-defined two-bond ($^2J_{CP}$) and three-bond ($^3J_{CP}$) carbon–phosphorus couplings in the $^{13}C$ NMR spectra (Murari et al., 1982), and the same was ob-

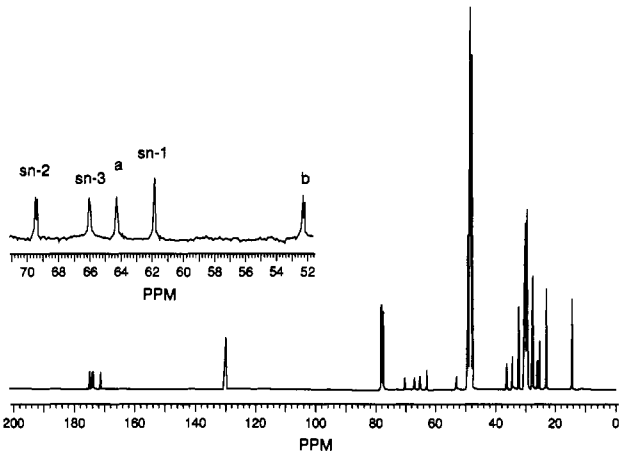


FIGURE 1: $^{13}C$ NMR spectrum of *N*-acyl-PS [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)serine]. Conditions as in Table I.

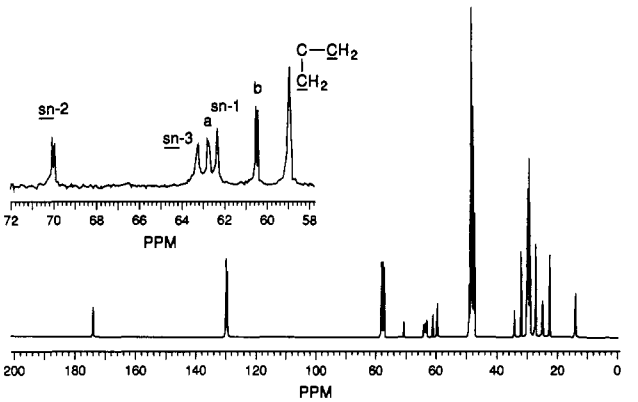


FIGURE 2: $^{13}C$ NMR spectrum of phosphatidyl-Tris (1,2-dioleoyl-*sn*-glycero-3-phospho-Tris). Conditions as in Table I.

Chart I

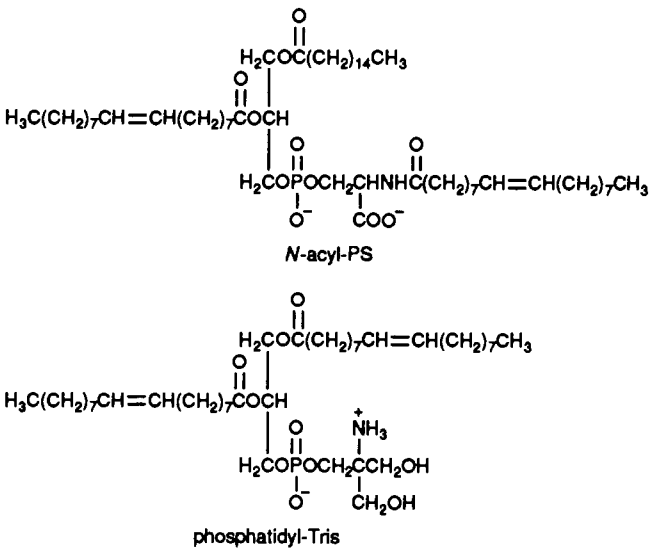

Table I: Carbon-13 NMR Data of *N*-Acyl-PS and Phosphatidyl-Tris

| carbon atom[a] | *N*-acyl-PS | phosphatidyl-Tris |
|---|---|---|
| *sn*-1 | 61.74 | 62.27 |
| *sn*-2 | 69.29 ($^3J_{CP}$ = 8.06 Hz) | 79.88 ($^3J_{CP}$ = 8.06 Hz) |
| *sn*-3 | 65.96 ($^2J_{CP}$ = 4.04 Hz) | 63.20 ($^2J_{CP}$ = 4.00 Hz) |
| $POCH_2$ | 64.14 ($^2J_{CP}$ = 4.03 Hz) | 62.69 ($^2J_{CP}$ = 3.90 Hz) |
| $POCH_2C$ | 52.15 ($^3J_{CP}$ = 8.06 Hz) | 60.61 ($^3J_{CP}$ = 6.04 Hz) |
| $HOCH_2C$-$CH_2OH$ | | 58.90 |
| $\alpha$ | 33.58, 33.50 | 33.63, 33.50 |
| $\alpha'$ | 35.39 | |
| $\beta$ | 24.32 | 24.32 |
| $\beta'$ | 25.06 | |
| $\omega - 2$ | 31.37 | 31.34 |
| $\omega - 1$ | 22.10 | 22.07 |
| $\omega$ | 13.37 | 13.34 |
| $(CH_2)_n$ | 28.8 | 28.74 |
| CH=CH | 129.44, 129.39, 129.17, 129.12 | 129.41, 129.11 |
| $CH_2CH$=$CHCH_2$ | 26.64 | 26.61 |
| C=O | 174.38, 173.41, 173.03, 170.65 | 173.7, 173.4 |

[a] $\alpha$ and $\beta$ refer to the first and second carbon atoms from the ester carbonyls and $\alpha'$ and $\beta'$ to the first and second carbon atoms from the amide carbonyl; $\omega$ refers to the terminal methyl carbon atoms. Spectra (75.429 MHz) were recorded in $CDCl_3$–$CD_3OD$–$D_2O$ (50:50:15 v/v) at a sample concentration of 20–30 mg/mL.

served for the polar head group and glycerol backbone carbon atoms of *N*-acyl-PS and phosphatidyl-Tris. The assignments and coupling constants given in Table I are in agreement with the observations of Murari et al. (1982) on similar phospholipids. In the case of *N*-acyl-PS, four peaks are observed between 170 and 175 ppm, and these were assigned to the four carbonyl groups of *N*-acyl-PS (see Figure 1). Phosphatidyl-Tris, which contains only two carbonyl groups, gives rise to only two peaks between 170 and 175 ppm (see Figure 2). Another important difference between the two spectra presented in Figures 1 and 2 is the position of the $POCH_2C$ carbon atom. In *N*-acyl-PS, the peak due to this carbon atom appears at 52.15 ppm because of the presence of an adjacent *N*-acyl, whereas it is shifted downfield at 60.61 ppm in the case of phosphatidyl-Tris. Also, the $\alpha'$ (35.39 ppm) and $\beta'$ (25.06 ppm) carbon atoms adjacent to the carbonyl of the *N*-acyl chain in *N*-acyl-PS are well separated from the corresponding $\alpha$ (~33.5 ppm) and $\beta$ (24.32 ppm) carbon atoms of the *O*-acyl chains at the *sn*-1 and *sn*-2 positions of the glycerol backbone. As expected, the $\alpha'$ and $\beta'$ carbon atoms are absent in the spectrum of phosphatidyl-Tris (Figure 2).

Infrared spectra of the two phospholipids are shown in Figure 3, representing *N*-acyl-PS (A) and phosphatidyl-Tris (B), and were interpreted according to established criteria (Bellamy, 1958; Kates, 1977). Characteristic absorption bands for *N*-acyl-PS are the C=O stretch of the secondary amide at 1680–1630 $cm^{-1}$ and the NH deformation at 1550–1510 $cm^{-1}$. Other prominent absorption bands, common to the IR spectra of both phospholipids, are the CH stretching of $CH_2$ at 2926 and 2853 $cm^{-1}$, the C=O stretch of the ester at 1750–1730 $cm^{-1}$, and the CH bending of $CH_3$ at 1450–1375 $cm^{-1}$.

In contrast, absorption bands in the spectrum of phosphatidyl-Tris are indicative of a primary amine at 3500–3300 $cm^{-1}$ (NH stretch) and 1650–1590 $cm^{-1}$ (NH deformation). Additional absorption bands attributable to the amino group are centered at 2600, 2090, 1560, and 1180 $cm^{-1}$. Absorption bands between 1000 and 1200 $cm^{-1}$ reflect C–O stretch frequencies of the alcohol groups in the Tris part of the molecule, and the presence of primary hydroxyl groups is also evident from the (hydrogen-bonded) O–H stretching at 3400–3200

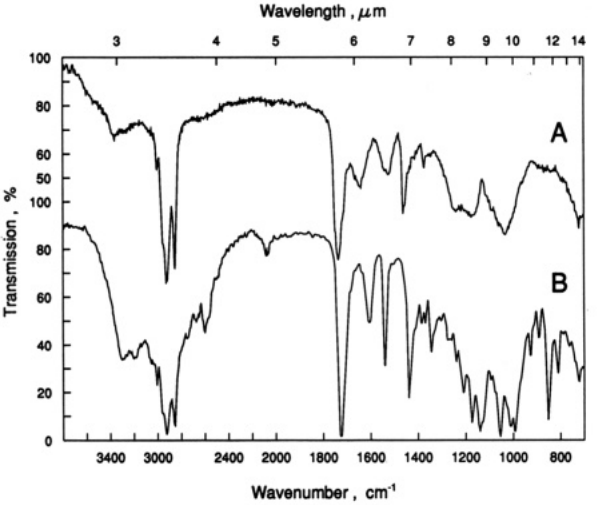


FIGURE 3: Infrared spectra of *N*-acyl-PS (A) and phosphatidyl-Tris (B).

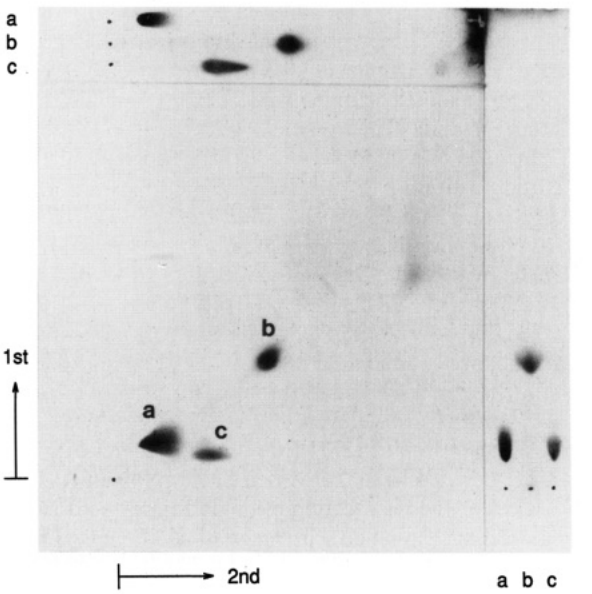


FIGURE 4: Two-dimensional thin-layer chromatogram of phosphatidyl-Tris (A), *N*-acyl-PS (B), and PS (C) on layers of silica gel G (Merck) containing 0.4 M boric acid, developed in the first direction with chloroform/methanol/water/ammonium hydroxide (70:30:3:2 v/v) and in the second direction with chloroform/methanol/water (65:35:5 v/v).

$cm^{-1}$, partially superimposed on the NH stretch of the primary amine.

The migration rates of the two phospholipids in thin-layer chromatography, relative to PS, are illustrated in Figure 4. On boric acid containing layers of silica gel G, *N*-acyl-PS migrated ahead of both PS and phosphatidyl-Tris with both alkaline and neutral developing solvents. Phosphatidyl-Tris and PS exhibited identical $R_f$ values with the alkaline solvent, whereas PS migrated ahead of phosphatidyl-Tris when the neutral developing solvent was employed. Neither phospholipid gave a color reaction with ninhydrin spray reagent, but phosphatidyl-Tris formed a fluorescent adduct with fluorescamine (Schmid et al., 1981), indicating the presence of a primary amine.

Phosphatidyl-Tris was completely deacylated by both alkaline and acidic methanolysis, whereas *N*-acyl-PS was fully deacylated only by strong acid methanolysis. Molar ratios of fatty acid:phosphorus obtained after alkaline and acid methanolysis are given in Table II. Alkaline methanolysis of

Table II: Acyl:Phosphorus Ratios of Synthetic *N*-Acyl-PS and Phosphatidyl-Tris[a]

| *N*-acyl-PS | | phosphatidyl-Tris | |
|---|---|---|---|
| | Alkaline Methanolysis | | |
| 16:0/P | 0.92, 0.90 | 18:1/P | 2.06, 1.98 |
| 18:1/P | 0.99, 0.96 | | |
| (16:0 + 18:1)/P | 1.91, 1.86 | | |
| | Acidic Methanolysis | | |
| 16:0/P | 0.93, 0.93 | 18:1/P | 1.81, 1.94 |
| 18:1/P | 1.86, 1.90 | | |
| (16:0 + 18:1)/P | 2.79, 2.83 | | |

[a] 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)serine and 1,2-dioleoyl-*sn*-glycero-3-phospho-Tris were subjected to alkaline and acidic methanolysis. Phosphorus and methyl esters were assayed as described under Materials and Methods. Data represent results of duplicate determinations.

*N*-acyl-PS yielded fatty acid methyl esters plus glycerophospho(*N*-acyl)serine. The latter was extractable into the organic phase of a lipid extract (Folch et al., 1957) and exhibited a $R_f = 0.12$ in thin-layer chromatography on silica gel H using chloroform/methanol/ammonium hydroxide/water (65:35:5:1 v/v) as developing solvent.

*Comparison of Synthetic Phospholipids with R. sphaeroides Phospholipid.* Donohue et al. (1982a) reported the isolation of a phospholipid from *R. sphaeroides* grown in Tris-containing media. The lipid was obtained by column chromatography in milligram quantities, further purified by preparative thin-layer chromatography, characterized chemically and spectroscopically, and assigned the structure of *N*-acyl-PS. The authors observed several unusual properties, including the fact that this lipid was eluted from DEAE-cellulose in high yield with chloroform/acetic acid (3:1), which is expected to elute only weakly acidic materials (Rouser et al., 1963, 1969). It should be noted that *N*-acyl-PS is an acidic phospholipid whereas phosphatidyl-Tris is zwitterionic. They also found that mild alkaline hydrolysis led to (almost) complete deacylation. This was explained by the potential destabilization of the serine *N*-acyl linkage by the proximity of the carbonyl group, thus making it susceptible to nucleophilic attack under alkaline conditions. They stated that synthetic *N*-acyl-PS was also completely deacylated by mild alkaline hydrolysis. However, the synthetic product had been obtained in only 5% yield after purification by thin-layer chromatography, and no chemical or physical characteristics for this material were reported. Both the synthetic material and the phospholipid isolated from *R. sphaeroides* were reported to be difficult to separate from PS by TLC (Donohue et al., 1982a). However, genuine *N*-acyl-PS is considerably less polar than PS due to the additional acyl group, and the two phospholipids are easily separable (Figure 4). In contrast, phosphatidyl-Tris remained near the origin in the two-dimensional thin-layer chromatography system used by Donohue et al. (1982a) and in the present study. Although phosphatidyl-Tris and PS comigrate on boric acid containing silica gel G using an alkaine solvent system, these lipids are separable by the use of a neutral developing solvent.

Our assumption that the polar lipid of *R. sphaeroides* consisted primarily of phosphatidyl-Tris is fully supported by its infrared spectrum (Donohue et al., 1982a), which is identical with that shown in Figure 3B (phosphatidyl-Tris) but different from the spectrum of *N*-acyl-PS (Figure 3A). Mild alkaline hydrolysis of phosphatidyl-Tris but not of *N*-acyl-PS would have yielded the complete deacylation reported by Donohue et al. (1982a), but the acyl:phosphorus ratio should have been 2 rather than the reported "approximately" 3, which

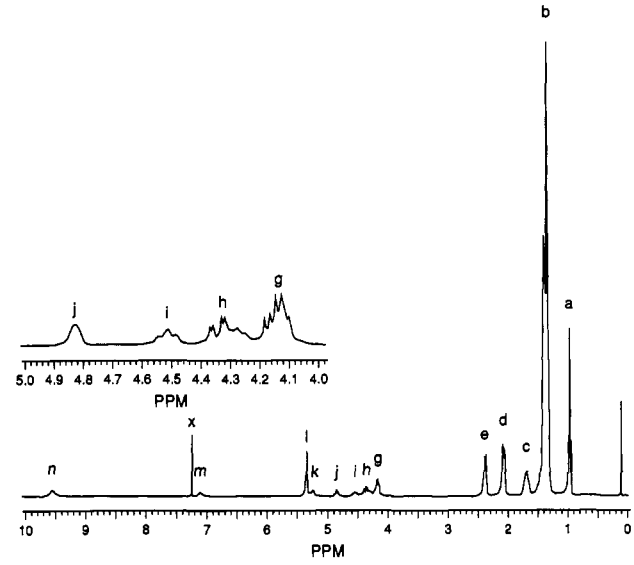


FIGURE 5: Proton NMR spectrum of *N*-acyl-PS [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)serine]. Conditions as in Table III.

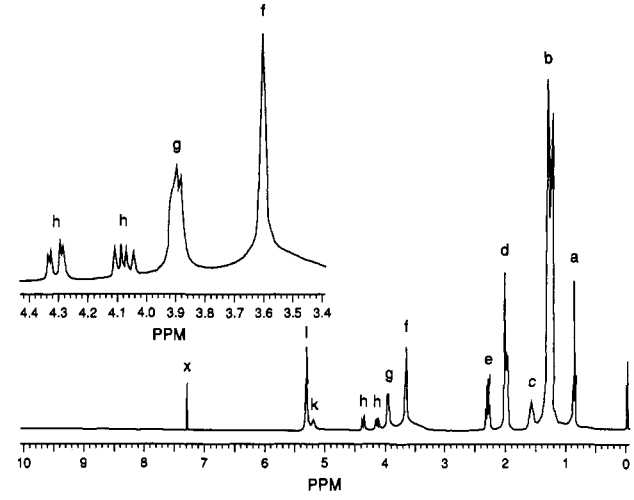


FIGURE 6: Proton NMR spectrum of phosphatidyl-Tris (1,2-dioleoyl-*sn*-glycero-3-phospho-Tris). Conditions as in Table III.

was based on colorimetric estimation of acyl groups.

Donohue et al. (1982a) also reported $^1$H NMR evidence to support the proposed *N*-acyl-PS structure. However, our $^1$H NMR spectra clearly show that the published spectrum of the *R. sphaeroides* phospholipid was actually of phosphatidyl-Tris, not *N*-acyl-PS.

The $^1$H NMR spectra of the two synthetic phospholipids are shown in Figures 5 and 6. Although the spectra are quite similar, they are distinctly different in the position of the four proton groups shown in the inserts. In the case of *N*-acyl-PS, these groups are seen between 4.0 and 4.9 ppm, whereas in the spectrum of phosphatidyl-Tris they occur between 3.6 and 4.4 ppm, which are the same positions reported by Donohue et al. (1982a). Chemical shift data for both phosphatidyl-Tris and *N*-acyl-PS are summarized in Table III. They agree well with assignments made by Hauser et al. (1988) for the $^1$H NMR spectrum of PS. Specifically, Hauser et al. observed the shift for $CH_2OCO$ at 4.36 ppm (a quartet of an eight-line multiplet corresponding to the AB part of an ABX system) and that of $CH_2OP/POCH_2$ at 4.24 ppm, whereas Donohue et al. assigned the peak at 4.16 ppm to $CH_2OCO$ and peaks at 3.74 ppm and 3.98 to $CH_2OP$ and $POCH_2$, respectively, for a total of 8 protons instead of 4. It should be noted also

Table III: Proton NMR Data of *N*-Acyl-PS and Phosphatidyl-Tris[a]

| | | *N*-acyl-PS | | phosphatidyl-Tris | |
|---|---|---|---|---|---|
| peak(s) | class of proton(s) | chemical shift (ppm) | no. of protons | chemical shift (ppm) | no. of protons |
| a | $t$-$CH_3$ | 0.88 | 8 | 0.87 | 6 |
| b | $(CH_2)_n$ | 1.27 | 61 | 1.27 | 40 |
| c | $COCH_2CH_2$ | 1.61 | 6 | 1.58 | 5 |
| d | $CH_2CH{=}CHCH_2$ | 2.01 | 8 | 2.01 | 8 |
| e | $COCH_2$ | 2.32 | 6 | 2.29 | 4 |
| f | $CH_2C(CH_2)CH_2$ | | | 3.69 | 5 |
| g | $POCH_2/CH_2OP$ | 4.15 | 4 | 3.97[b] | 2 |
| h | $CH_2OCO$ | 4.32 | 2 | 4.37, 4.07 | 2 |
| i | $CHNH$ | 4.51 | 1 | | |
| j | NH | 4.83 | 1 | | |
| k | CHOCO | 5.24 | 1 | 5.21 | 1 |
| l | CH=CH | 5.33 | 4 | 5.33 | 4 |
| m | POH | 7.15 | | | |
| n | COOH | 9.60 | | | |
| x | $CHCl_3$ (residual) | 7.26 | | 7.26 | |

[a] Spectra (300 MHz) were recorded in deuterated chloroform at a sample concentration of 20–30 mg/mL. Chemical shift values refer to tetramethylsilane set at 0 ppm. Peaks a–o refer to Figures 5 and 6. [b] $CH_2OP$ only.

that substitutions at the polar head group do not appear to affect the position of the $CH_2OCO$ peak, since in PS, *N*-acyl-PS, and phosphatidyl-Tris (Table III) this peak is found at ~4.35 ppm. We therefore have identified the four groups of protons in the spectrum of phosphatidyl-Tris as $CH_2C(CH_2)CH_2$ (3.69 ppm), $CH_2OP/POCH_2$ (3.97 ppm), and $CH_2OCO$ (4.07, 4.37 ppm). These shifts closely correspond to those reported by Donohue et al. (1982a), namely, 3.74, 3.98, 4.16, and 4.36, respectively. Our data show 61 methylene protons for synthetic *N*-acyl-PS but only 40 for phosphatidyl-Tris.

In summary, all available information indicates that the polar phospholipid isolated from *R. sphaeroides* grown in Tris-containing media is primarily and very likely exclusively phosphatidyl-Tris. Due to the similarity in chromatographic properties, the isolated material may have contained a small amount of PS which would explain the presence of serine in the hydrolysate, as evidenced by paper chromatography or the use of an amino acid analyzer (Donohue et al., 1982a).

## Discussion

To establish the molecular structure of a novel phospholipid is notoriously difficult, even if the lipid is obtained in high purity and in sufficient quantity for characterization. Elemental analyses and chemical degradation can be inconclusive, migration rates in chromatography and acyl:phosphorus ratios can be quite variable under different experimental conditions, and spectroscopic evidence is reliable only in comparison to authentic standards. Donohue et al. (1982a) have published complete IR and $^1H$ NMR spectra of the *R. sphaeroides* phospholipid which, taken together with other evidence, is sufficient for structure identification provided that proper synthetic standards are used. We have synthesized and characterized both *N*-acyl-PS and phosphatidyl-Tris. Comparison of the chemical and chromatographic properties of these two phospholipids, as well as their IR and $^1H$ NMR spectra, with the data reported for the *R. sphaeroides* phospholipid (Donohue et al., 1982a) establishes the identity of this lipid as phosphatidyl-Tris rather than *N*-acyl-PS. Because the *R. sphaeroides* phospholipid was shown to be considerably more polar than authentic *N*-acyl-PS, we believe that there exists no evidence for the presence, even in small amounts, of *N*-acyl-PS in organisms grown either in Tris-supplemented or Tris-free media. The small amounts of "*N*-acyl-PS" observed in these cells grown in Tris-free media (Donohue et al., 1982a) were probably PS or some other polar phospholipid (Lascelles & Szilagyi, 1965).

It is therefore necessary to reinterpret the results of a number of experiments dealing with "*N*-acyl-PS accumulation" in *R. sphaeroides*. Rather than being indicative of a novel pathway of phospholipid synthesis, the accumulation of this phospholipid must be due to an uptake of Tris from the media into *R. sphaeroides* followed by its utilization as a substrate for phospholipid biosynthetic enzymes. At this time, the mechanism of incorporation of Tris into phosphatidyl-Tris remains obscure, but the synthesis of unusual phosphatidic acid derivatives was reported for other systems. *Escherichia coli* was shown to synthesize phosphatidylmannitol and diphosphatidylmannitol when grown in 600 mM mannitol (Shibuya et al., 1985), and the GDP-mannose:dolichol-phosphate mannosyl transferase of hamster liver can apparently transfer mannose to phosphatidic acid yielding phosphatidylmannose (Creek et al., 1986).

Our present results may be important in future studies for two reasons. One is the fact that caution should be exercised when Tris buffer is used in vivo or in vitro in experiments involving *R. sphaeroides* or other organisms that can utilize Tris for phospholipid synthesis. Second, it should be of interest whether and to what extent phosphatidyl-Tris is incorporated into the membranes of *R. sphaeroides* or other organisms. It has been shown that this lipid can represent up to 40% of total lipid phosphorus without affecting growth of the organism (Donohue et al., 1982b). This suggests that membranes may be able to accommodate massive amounts of a "foreign" phospholipid without becoming functionally impaired. Finally, it is interesting to note that certain strains of *R. sphaeroides*, including wild-type 2.4.1 and RS2, can successfully discriminate against Tris (Donohue et al., 1982b). This should make it possible to define the enzyme or enzymes that allow Tris to enter phospholipid biosynthesis.

**Registry No.** *N*-Acyl-PS, 131180-30-8; phosphatidyl-Tris, 131235-28-4.

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# Effects of Detergent Environments on the Photocycle of Purified Monomeric Bacteriorhodopsin†

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ABSTRACT: Time-resolved difference spectra have been obtained for the photocycle of delipidated bacteriorhodopsin monomers (d-BR) in six different detergent micelle environments that were prepared by two new detergent-exchange techniques. A global kinetic analysis of the photocycle spectra for d-BR in each detergent environment was performed. Comparison of these results with those obtained for the photocycle of bacteriorhodopsin in purple membrane (PM) shows that there is one fewer kinetically distinguishable process for monomeric BR between the decay of the K intermediate and the rise of the M intermediate. Assuming a sequential pathway occurs in the photocycle, it appears that the equilibrium between the L and M intermediates is reached much more rapidly in the detergent micelles. This is attributed to a more direct interaction between Asp-85 and the proton on the nitrogen of the Schiff base of retinal for BR in the detergents. Equilibrium concentrations of late photocycle intermediates are also altered in detergents. The later steps of the photocycle, including the decay of the M intermediate, are slowed in detergents with rings in their hydrocarbon region. This is attributed to effects on conformational changes occurring during the decay of M and/or other later photocycle intermediates. The lifetime of dark adaptation of light-adapted d-BR in different detergent environments increases in environments where the lifetime of the M intermediate increases. These results suggest that the high percentage of either unsaturated or methyl-branched lipids in PM and the membranes of other retinal proteins may be important for their effective functioning.

Bacteriorhodopsin (BR)[1] is the primary protein found in the cell membrane of *Halobacterium halobium*. Absorption of visible light by the chromophore of BR, a protonated Schiff base of retinal, initiates a photocycle whose function is to pump one or more protons across the cytoplasmic membrane (Ottolenghi, 1982; Stoeckenius & Bogomolni, 1982; Ottolenghi & Sheves, 1989). A large number of studies have been reported on how light absorption by BR eventually leads to the transfer of a proton across the membrane.

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[1] Abbreviations: BR, bacteriorhodopsin; $C_{12}E_8$, octylethylene glycol dodecyl ether; d-BR, delipidated BR monomers; d-BR/CHAPSO, d-BR in 16 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; d-BR/NG, d-BR in 18 mM nonyl β-D-glucopyranoside; DDM, dodecyl β-D-maltoside; HTX, hydrogenated Triton X-100; NG, nonyl β-D-glucopyranoside; PM, purple membrane; TX, Triton X-100.

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